EXHIBIT 22

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Microtiter plate assay for measuring the anticomplementary activity of immunoglobulins

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An anticomplementary activity (ACA) assay on a microtiter plate combined with a computer-controlled plate reader and computerized calculation of the assay result is described. The assay is a simplified version of existing ones and can be used to assay immunoglobulins at low protein concentrations. The method detects the consumption of 0.13 $CH_{50}U$, corresponding to anticomplementary activity of 7.5 $CH_{50}U/g$ protein. The assay has a coefficient of variation of 20.4%.

Key words: Anticomplementary activity; Intravenous immunoglobulin; Complement activity

Introduction

Over the past 20 years a succession of intravenous immunoglobulin preparations have been introduced into clinical practice. One of the key issues for the safety of an intravenous immunoglobulin preparation is a low or absent ability to activate complement in vivo via the classical pathway independent of antigen. The quality of an intravenous immunoglobulin preparation is reflected by the degree of nonspecific activation of complement, the so-called anticomplementary activity (ACA). High ACA is associated with a high content of aggregates in the preparation or with damage to the Fc portion of the molecule, and such a product, when infused, causes serious side effects, and often an anaphylactic reaction.

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The activity of complement is measured in vitro, using guinea pig complement and sheep red cells sensitized with rabbit antibodies against sheep red cells as originally described by Von Krogh (1916). Recently, the principle has been adapted to measure the level of complement in sera using microtiter plates (Blann et al., 1990), and the results can be calculated by computer (Blann et al., 1990; Kamiyama et al., 1990).

Methods of assaying ACA in vitro have been published by Miekka and Gozze (1975) and Römer et al. (1979). After a defined amount of immunoglobulin has been incubated with a defined amount of complement, the residual complement activity is titrated, employing sheep red cells coated with antibody. The published methods for such measurements of ACA are not designed to meet the needs of product development. Here we present a microtiter plate assay of ACA which is simple, rapid and easily adapted to large number of samples. To establish the sensitivity and reproducibility of this method, we as-

sayed samples of commercially available intravenous (i.v.) and intramuscular (i.m.) immunoglobulin preparations. Our results are in agreement with those of other ACA assays.

Materials and methods

Principle of the assay

A defined amount of immunoglobulin is incubated with a defined amount of complement and the remaining complement is titrated with sheep red cells coated with antibody. The complement consumption capacity is calculated as the difference between the residual activities in the sample and the buffer control, and is expressed as complement hemolyzing units ($CH_{50}U$) per gram of protein. The complement hemolyzing unit (1 $CH_{50}U$) is the amount of complement which has the capacity to hemolyse 2.5×10^8 sensitized sheep red cells (Mayer, 1961). Anticomplementary activity is represented by the following equation: ACA in $CH_{50}U/g = (C_1 - C_0)/P$, where C_1 = the amount of complement in $CH_{50}U$ bound by the sample, C_0 = the amount of complement in $CH_{50}U$ bound by the buffer control, and P = the amount of protein in the sample in grams.

Reagents and equipment

Anti-sheep red blood cell (SRBC) serum (Amboceptor 6000, ORLC 24/25) and guinea pig complement (ORAY 20/21) were obtained from Behringwerke (Marburg, Germany). These reagents were dissolved, dispensed as aliquots, and stored frozen at −70°C. Aliquots were not refrozen. Fresh sheep blood, collected in Alsever anticoagulant, was obtained from the National Public Health Laboratory, Helsinki. Preserving solution for red cells (PSRC) consisted of 72 mM NaCl, 20.5 g/l glucose, 2 g/l inosine, 0.33 g/l chloramphenicol, and 30 mM trisodium citrate; the pH was adjusted with NaOH to 6.8. The working buffer (WB) consisted of 154 mM NaCl, 80 g/l sucrose, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH adjusted with HCl to 7.3. The gelatine-veronal buffer (GVB) consisted of 1.0 g/l gelatine, 154 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 11 mM veronal, pH 7.3.

TABLE I

IMMUNOGLOBULIN PREPARATIONS TESTED

No.	Code	Origin of the sample	Type	Protein concentration in the assay (g/l)	Number of replicates (n)	Consumption of complement		Anticomplementary activity	
						Mean (μl)	SD (μl)	Mean ($CH_{50}U/g$)	SD ($CH_{50}U/g$)
1	14341001	FRC BTS	i.v.	60	12	46.6	10.0	2.2	2.8
2	14341002	FRC BTS	i.v.	60	5	46.3	4.3	3.4	2.2
3	14341003	FRC BTS	i.v.	60	35	63.2	22.6	8.5	10.3
4	14341004	FRC BTS	i.v.	60	24	56.5	19.9	4.7	9.4
5	0.521.017.0	SRC BTS	i.v.	50	20	57.1	20.9	7.3	11.5
6	0.571.015.0	SRC BTS	i.v.	60	22	56.9	10.5	5.9	4.1
7	Sandoglobulin	SRC BTS	i.v.	60	12	49.8	6.8	5.2	3.3
8	Endobulin	Immuno	i.v.	50	27	60.5	28.7	9.5	15.7
9	Gammagard	Travenol	i.v.	50	24	97.0	46.3	25.0	22.9
10	Venimmun	Behring	i.v.	50	32	61.5	25.0	11.1	13.6
11	Gammavenin	Behring	i.v.	50	26	42.8	7.0	2.7	3.0
12	Gammonativ	Kabi	i.v.	60	24	81.1	33.3	16.5	16.7
13	11111011/11	FRC BTS	i.m.	16	20	105.4	38.9	39.1	22.8
14	66704.51	Kabi	i.m.	16	24	87.1	26.9	28.1	15.3
15	Beriglobin	Behring	i.m.	5	14	56.6	8.7	65.2	57.4

EDTA-GVB consisted of 1.0 g/l gelatine, 154 mM NaCl, 10 mM EDTA, and 11 mM veronal, pH 7.3.

Microtiter plates were from Nunc (Denmark), the automatic diluter was a Microlab 1000 (Hamilton, USA), the plate reader was a Multiscan MCC (Labsystems Oy, Finland), and the software for controlling the plate reader and calculating the results was a Flexicalc (Wallac Oy, Finland). The computer was an ALR Powerflex Plus with a 386 SX processor.

Preparation of sensitized sheep red cells

Anticoagulated sheep blood was centrifuged at $3350 \times g$ for 10 min. The plasma and buffy coat were discarded. The red cells were suspended in 0.9% saline and centrifuged again at $3350 \times g$ for 5 min. Washing was continued with 0.9% saline ($\times 1$) and with EDTA-GVB solution ($\times 1$). The cell density of the suspension was adjusted to 10^9 cells/ml. A dilution of 1/15 in distilled water was made to hemolyse the cells, and the absorbance of the hemoglobin solution was measured at 541 nm. The amount of EDTA-GVB solution required for this adjustment was calculated from the equation: $L_1 = S_1 \times (A_1 - 0.7)/0.7$, where L_1 = amount of EDTA-GVB solution required (ml), S_1 = volume of cell suspension (ml) and A_1 = measured absorbance of the hemolysate at 541 nm. The working dilution of anti-SRBC serum, (usually diluted 1/1000 with EDTA-GVB) and an equal volume of cell suspension containing 10^9 cells/ml were mixed and incubated for 15 min in a water bath at 37°C. The cells were washed twice with 0.9% saline, centrifuged at $3350 \times g$ for 5 min, and resuspended in PSRC to be stored as a 5% (v/v) stock suspension. Washed cells were stored in a refrigerator and used for up to 6 weeks. If hemolysis or aggregation was observed, the cells were not used in the test.

Samples

The samples and the protein concentrations used in the assay are listed in Table I. There is no international immunoglobulin reference preparation available for the ACA test. Dr. A. Gardi, Central Laboratory of the Swiss Red Cross, Bern, provided samples 5 and 6 used in his laboratory and Dr. P. Gronski provided samples 10 and 11 used in Behringwerke AG, Marburg, as references. Other preparations studied were either standard immunoglobulin preparations for intravenous use (Sandoglobulin (SRC BTS, Switzerland), Gammonativ (Kabi, Sweden), Endobulin (Immuno, Austria), and Gammagard (Travenol, USA)), and a new i.v. immunoglobulin preparation which is solvent/detergent-treated (Venogamma, Finnish Red Cross, Blood Transfusion Service (FRC BTS, license pending), or were immunoglobulins for intramuscular use (Beriglobin (Behringwerke AG) and intramuscular immunoglobulins from FRC BTS and Kabi). The samples were dissolved according to the instructions of the manufacturers, divided into small aliquots, and stored frozen at −20°C. In the assay, the protein concentration of the sample was adjusted, depending on the level of anticomplementary activity of the sample.

Assay

The samples and the amount of complement needed were thawed in a water bath at 37°C. Aliquots of complement solution (40 μl), sample (300 μl), and WB (1460 μl) were mixed in Wassermann tubes, using the Microlab 1000 diluter, and incubated for 1 h in a water bath at 37°C. A buffer control was included in each assay. It consisted of complement solution (40 μl) and WB (1760 μl). After the consumption step, a series of increasing amounts of the above mixtures, from 14 to 124 μl, were pipetted in duplicate into the wells of a flat-bottomed 96-well microtiter plate with the diluter. In order to monitor the level of hemolysis, residual cell controls of maximal (T) and zero response (B) were included in each plate in the assay. 100 μl 0.1% sodium dodecyl sulfate (SDS) were pipetted into residual cell controls with zero response (B). Fig. 1 shows the layout of the pipetting order. A sensitized SRBC suspension (200 μl) was added to all wells and the plate was incubated for 1 h at 37°C. The cell concentration was selected so that after hemolysis the mean absorbance of the residual cell controls with maximal response (T) was approximately 1.0. This corresponds to 1.12×10^8 cells per well. To adjust the density to 5.6×10^8 cells/ml, the cell suspension was diluted 1/15

	1	2	3	4	5	6	7	8	9	10	11	12
A	I 1	I 1	II 1	II 1	III 1	III 1	IV 1	IV 1	V 1	V 1	VI 1	VI 1
B	I 2	I 2	II 2	II 2	III 2	III 2	IV 2	IV 2	V 2	V 2	VI 2	VI 2
C	I 3	I 3	II 3	II 3	III 3	III 3	IV 3	IV 3	V 3	V 3	VI 3	VI 3
D	I 4	I 4	II 4	II 4	III 4	III 4	IV 4	IV 4	V 4	V 4	VI 4	VI 4
E	I 5	I 5	II 5	II 5	III 5	III 5	IV 5	IV 5	V 5	V 5	VI 5	VI 5
F	I 6	I 6	II 6	II 6	III 6	III 6	IV 6	IV 6	V 6	V 6	VI 6	VI 6
G	T	T	T	T	T	T	T	T	T	T	T	T
H	B	B	B	B	B	B	B	B	B	B	B	B

Fig. 1. Layout of samples and controls on a microtiter plate. Sample, complement, and buffer are incubated together and a series of increasing amounts of the mixture is dispensed in duplicate into the wells. The corresponding volumes of the aliquots are: 1 = 14 μl, 2 = 36 μl, 3 = 58 μl, 4 = 80 μl, 5 = 102 μl and 6 = 124 μl. The wells marked I are complement challenged with buffer and wells marked II through VI are complement challenged with appropriate dilutions of samples. Residual cell controls of maximal (T) and zero response (B) are included in each plate. The assay continues as described in the materials and methods section.

with distilled water and the absorbance of the hemoglobin solution was measured at 541 nm. The amount of GVB required was calculated from the equation: $L_2 = S_2(A_2 - 0.35)/0.35$, where L_2 = amount of GVB required (ml), S_2 = volume of cell suspension (ml) and A_2 = measured absorbance of hemolysate at 541 nm. After incubation the residual cells were centrifuged at $500 \times g$ for 5 min and the supernatants from each well were decanted and discarded. The nonhemolysed cells were further washed twice with 0.9% saline and subsequently hemolysed by adding 200 μl 0.1% SDS solution and incubated for about 1 h at 37°C. In the course of incubation, the plates were occasionally stirred carefully. The absorbances of the wells were measured at 541 nm with the Multiscan MCC plate reader.

The Flexicalc computer program controlled the plate reader and the collection and storage of the raw data. The program then calculated the volume of complement needed for 50% hemolysis of each sample. According to Von Krogh (1916), the activity of complement is calculated as: $x = K(y/(1-y))^{(1/n)}$, where x = amount of complement, y = degree of hemolysis ($= (A - B)/(T - B)$), K = unit of complement activity which hemolyses 50% of the sensitized red cells under standard conditions, and n = a constant depending on the experimental conditions. Log-log transformation of the data gave a linear dose-response curve. A typical plot of an assay is shown in Fig. 2.

Results

The average activity of the complement preparation used throughout the study was calculated retrospectively. A buffer control was included in each plate in each assay session. The average volume that hemolysed 50% of the sensitized red

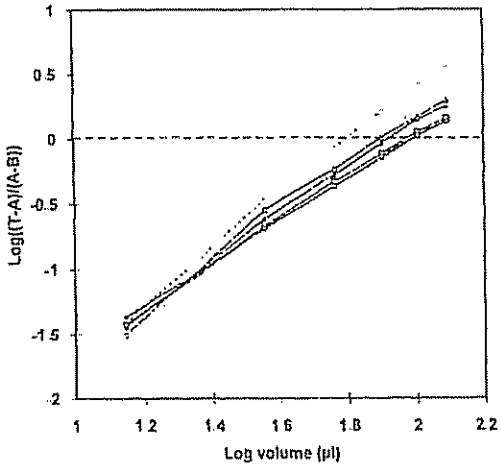

Fig. 2. Calculation of the residual volume of complement. A defined amount of complement solution was challenged with a defined amount of immunoglobulin in the sample as is shown in Table I. A constant amount (1.12×10^8 cells in 0.2 ml) of sensitized red cells were added to a series of increasing amounts of complement solution in duplicate, as shown in Fig. 1. Surviving red cells were separated from the hemolysate and the amount of cells was estimated by measuring the absorbance after subsequent hemolysis with detergent. The absorbance data were transformed according to Von Krogh's method (1916) and the value of the residual volume of complement corresponding to 50% hemolysis was calculated. The horizontal dashed line corresponds to 50% hemolysis. Linear regression was applied to construct the best fit for the transformed responses of each sample. The intersection of the response curve corresponding to the 50% hemolysis line gave the log value of the residual volume of complement. The topmost (dotted line) represents the buffer control.

cells in the series of 104 assays was calculated to be 46.5 μl (SD = 9.5 μl, CV = 20.4%). From the definition of the CH_{50} unit and the mean value (46.5 μl) for the consumption of complement, it can be concluded that the activity of the stock solution of the complement used was 217 CH_{50} units/ml. A small loss in the activity of complement was found after storage of aliquots for 10 months at −20°C. The series of assays were divided into three groups. The mean value for the consumption of complement by the buffer sample was 39.5 μl in the first group, 48.5 μl in the second group, and 62.6 μl in the third group. These differences were statistically significant (t test). Therefore in order to decrease the error caused by this factor, the complement consumption of the buffer samples in the assay results, which were collected over a period of 10 months, were separately calculated in three groups and the differences between the respective amounts of complement consumed by the buffer sample was taken into account.

The dose response of an intramuscular immunoglobulin preparation known to possess high anticomplementary activity was studied. The consumption of complement was measured at five protein concentrations and the amount of residual complement was calculated from the data of four sets of parallel measurements. In Fig. 3 the volumes of complement consumed (corresponding to 50% hemolysis) are plotted against the corresponding protein concentrations. A linear correlation ($r = 0.999$) between the protein concentration and the amount of complement consumed was observed when the consumption of complement was less than about 150 μl (0.71 CH_{50} units).

The average volume of complement consumed by the buffer controls ($n = 104$), corresponding to 50% hemolysis, was 46.5 μl (SD = 9.5 μl). Assuming a normal distribution for the variation in the assay results, the limit of detection at the 99.9% level of confidence was 75 μl (mean + 3 SD).

Immunoglobulin preparations from different manufacturers (listed in Table 1) were assayed, and the mean values and corresponding standard deviations are shown in Fig. 4. Two intravenous immunoglobulins and two of the three intramus-

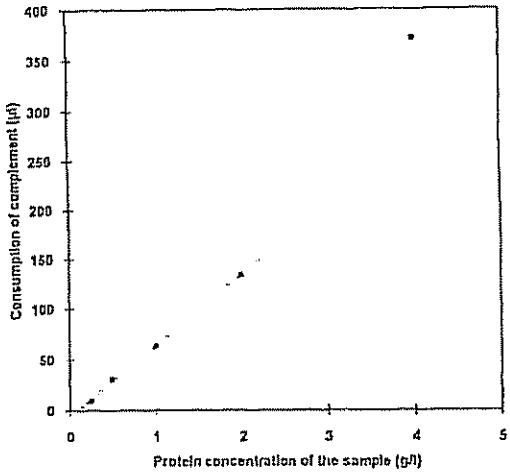

Fig. 3. Dose-response curve for an i.m. immunoglobin preparation. The delta consumption of complement is plotted as a function of the protein concentration in the sample. The points are average values of four measurements.

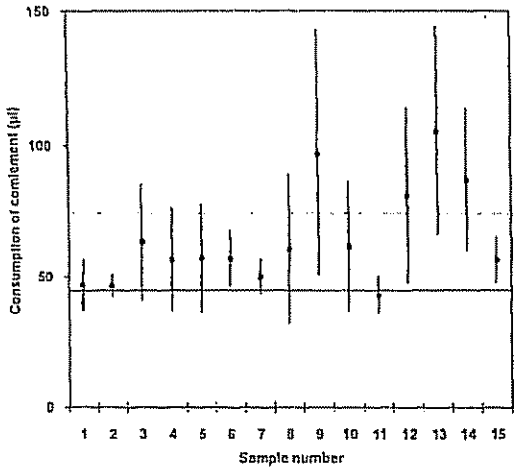

Fig. 4. Results of complement consumption of various immunoglobulin preparations. The amount of complement consumed by each of the immunoglobulin preparations is expressed as the average of replicates and the corresponding standard deviation. The figures and the sample identification are shown in Table I. The limit of detection, 75 μl (= mean of complement controls + 3 SD), is shown as a dotted line. Note that sample no. 15, which is highly anticomplementary, clearly deviates from zero, but is below the detection limit. The explanation for this is given in the text.

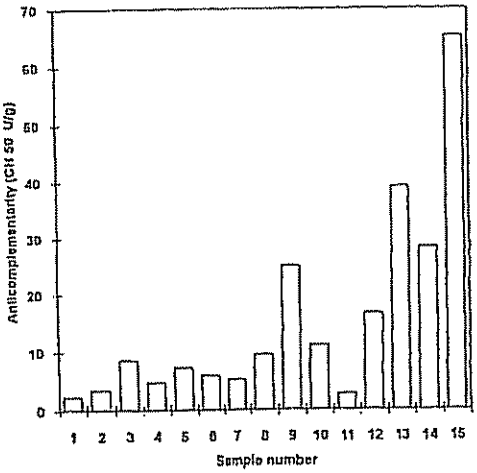

Fig. 5. Anticomplementary activity of various immunoglobulin preparations. Anticomplementary activity per gram of protein was calculated from the delta consumption of complement ($CH_{50}U$) in relation to the amount of protein of the sample in the test. The sample identification is shown in Table I.

cular preparations exhibited average complement consumption values which differed significantly from the average value of the buffer control. Ten intravenous immunoglobulin preparations yielded values which were clearly below the level of detection.

The anticomplementary activity of each immunoglobulin preparation was calculated from the average amount of complement consumed by the protein in the sample (Table I) and the results are shown in Fig. 5.

Discussion

Binding of complement by antigen-antibody complexes is essential for the humoral efficacy of immunoglobulin in vivo. However, if immunoglobulin binds complement in the absence of antigen, such an immunoglobulin may cause serious side effects when infused intravenously.

The ACA test is used by manufacturers of intravenous immunoglobulin preparations to screen the quality of their preparations, even though it is not clear how reliably the results correspond to the side effects. Titration of anticomplementary activity in patients is impossible for ethical reasons. However, clinical experience warns us not to use preparations with high ACA.

ACA measurement is included in the European Pharmacopoeia monograph recommending procedures to be adapted when using intravenous immunoglobulin (Ph. Eur., Anonymous, 1991). The assay modification presented here follows the guidelines for the proposed Ph. Eur. method. The original method of measuring complement activity described by Mayer (1961) and its modifications for assessing anticomplementary activity as referred to by Miekka and Gozze (1975) provided the basis for the present approach. We have compromised by assessing the amount of nonhemolysed red cells after challenging with various amounts of residual complement instead of measuring the degree of hemolysis directly. Using this modification we could measure the effect of complement directly in the well where the complement reaction was taking place, and transfer of the hemolysate to a second microtiter plate was not needed. Other improvements over previous methods mainly increased the capacity of the assay and reduced the clerical errors because the absorbances of the samples and the calculation of results were controlled by a computer.

In this study, the activity of the stock solution of complement was 217 $CH_{50}U/ml$ and this is in agreement with the declaration of the manufacturer. In the course of study we noticed a small change in the consumption of complement by buffer, but for routine purposes, this has no effect because each plate contains a buffer control.

Miekka and Gozze noted (1975) that serial dilution of a sample may increase the consumption of complement. We suggest that the enhancement of anticomplementary activity caused by dilution is due to changes in steric conformation of the immunoglobulin at low concentrations. However, no serial dilution procedure is employed in the present assay method.

Appropriate dilution is important in this procedure because the assay is linear only over a relatively narrow working range (about five-fold). The importance of dilution is illustrated by sample 15 (known to have a high ACA), which was diluted before assay to a protein content of 0.5%. The consumption of complement by this sample

was constantly below the detection limit (mean + 3 SD) of the assay. However, the mean value of complement consumed by this sample was different from that of the buffer control and when its protein content was taken into account, its anti-complementary activity proved to be the highest of all the samples studied. If there are too few results for statistical analyses, highly diluted samples will incorrectly appear to have low ACAs.

Because of the consumption of complement by the buffer samples, this assay detects 0.13 CH_{50} units, which in a 6% solution equals 7.5 $CH_{50}U/g$ protein. The ACA assay includes variables of biological origin. The sensitivity and precision of the assay depend not only on the complement preparation and the anti-SRBC serum but also on the concentration of each component. The origin of the sheep red cells may also have an influence; we have found some variation between individual sheep, which we have not yet studied in detail. However, the sheep red cells are not a major source of error in this test.

Because of the many variables, it may be assumed that assay results on the same preparations will differ from one laboratory to another and this emphasises the urgent need for an international reference preparation.

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EXHIBIT 23

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Anticomplementary Activity and the Safety of Intravenous Immunoglobulin

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ABSTRACT

Quality assurance release levels for anticomplementary (AC) activity of a nonmodified intravenous immunoglobulin (IGIV, pH 4.25) were reviewed over a period of one year and nine months in an attempt to correlate the incidence of complement-mediated-type adverse reactions with the AC level. Over 200 lots and ten possible complement-mediated side effects were evaluated. No correlation was found. A similar evaluation was made of a prospective study in which three lots of IGIV, pH 4.25 (one with a low, one with a mid-range, and one with a high AC activity) were used; again no correlation was found. Finally, a comparison was made between the AC activity assay used to release IGIV, pH 4.25 and an alternative AC activity assay designed for a highly modified IGIV preparation. It was demonstrated that the AC activity assay designed for the modified preparation and its assay limits were unsuitable in attempting to define AC activity of the nonmodified preparation.

INTRODUCTION

The ability to fractionate human blood plasma into its component parts[1-5] enabled Barandun and his colleagues[6] in 1962 to investigate patients' intolerance to intravenous administration of the human gamma globulin fraction. Using a standard gamma globulin preparation intended for intramuscular use, they showed that only 13% of subjects without antibody deficiency developed a reaction on intravenous administration, whereas 92% of patients with antibody deficiency developed a reaction. The investigators felt it was unlikely that the reactions seen were due to direct antigen-antibody reaction—the recipients' antibodies reacting with antigens present in the gamma globulin concentrate—since patients with immune deficiency are unable to form antibodies.

They suggested that the reactions could be due to antigens in the recipient organism reacting with antibodies of the administered gamma globulin preparation, or that certain gamma globulin prepara-

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tions or certain lots of such preparations might have an intrinsic ability to activate the complement system when given intravenously. The latter mechanism might be explained by aggregated gamma globulins which fix complement by a similar mechanism to antigen-antibody complexes. Measures that might be taken to treat the standard gamma globulin preparation and render it less likely to spontaneously fix complement in the absence of antigen were presented.

A major characteristic of current gamma globulin preparations that have been rendered safe for intravenous use is either minimal anticomplementary (AC) activity or the absence of AC activity.[7-9] It is axiomatic, therefore, that an appropriate test for AC activity is included in the quality assurance battery required for release of the different intravenous immunoglobulin preparations.

We reviewed the data of certain anticomplementary activity assays used to release an intravenous immunoglobulin rendered safe for intravenous infusion by stabilization at pH 4.25 (IGIV, pH 4.25).[10] The AC activity was correlated against complement-type mediated adverse reactions that were reported spontaneously or observed during clinical investigations. We also evaluated an alternative assay specifically developed to measure AC activity of nonmodified intravenous immunoglobulin (IGIV, pH 4.25) to determine whether this assay and its limits could be used to assess the safety of the preparation for intravenous administration.

PATIENTS AND METHODS

Each patient in this study received infusions of an intravenous immunoglobulin fractionated from human blood plasma by the Cohn-Oncley fractionation method and then stabilized in solution at a pH of 4.25.[10] The number of lots used during the period of spontaneous reporting exceeded 200 and were freely available on the market. Clinical product research (PR) lots were used for patients in the surveillance study.

Two groups of patients were examined for possible complement-mediated adverse reactions. All untoward effects of the first group (group 1) considered attributable to administration of IGIV, pH 4.25 were spontaneously reported to Cutter Biological between the period of March 1986 and December 1987.

The second group (group 2) was comprised of 30 patients at two centers participating in a surveillance study. All 30 patients had primary immune deficiency (14 patients with X-linked agammaglobulinemia, 14 patients with common variable agammaglobulinemia, and two patients with Wiskott-Aldrich syndrome). The patients were randomly allocated to receive monthly infusions (400 mg/kg) of IGIV, pH 4.25. Each patient received four infusions from each of three different lots. A change of lot was made after the first four months and again after the second four months, with the third lot being administered in the last four months. The three lots used were chosen deliberately to have a high AC activity (PR 3025), a mid-range AC activity (PR 3032), and a low AC activity (PR 3055), as determined by in vitro AC activity Method 1. The AC activity of all three lots was within the acceptable AC activity range for this assay. A total of 342 infusions were administered. The average infusion time was 1.68 ± 0.49 (SD) hours; the average dosage administered was 399 mg/kg and the average volume given was 401 ml.

Assays for Anticomplementary Activity

Method 1

Compliance with the AC activity specification in accordance with the Cutter Biological AC test is required for all lots of IGIV, pH 4.25 released to the market, as well as those used in the definitive surveillance study. This assay consists of addition of one volume of two CH_{50} units of guinea pig complement to an equal volume of sequential dilutions of test material in an appropriately buffered solution. The appropriate dilutions of hemolysin and complement are determined by titration prior to running the assay. The diluted test samples are allowed to react with complement for two hours at 37 °C. After incubation, sensitized sheep red blood cells are added and percent inhibition of hemolysis determined. The AC values are based upon the amount of test material that produces 50% inhibition of hemolysis. Reference standards are run with each assay to verify the performance of the test. The release limit for the test is less than 25 units CH_{50}/ml of product.

Method 2

For comparative purposes, complement assays were also performed on ten of the marketed lots, using a complement assay originally designed to assess complement activity of an intravenous immunoglobulin preparation rendered safe for intravenous administration by pepsin digestion. This assay uses one volume of 100 CH_{50} of guinea pig complement added to one volume of test material. Working dilutions of hemolysin and complement to be used in the assay are determined previously by titration. Three volumes of appropriate buffer are added to the two volumes and the material is allowed to react for one hour at 37 °C. A control is run in which the volume of test material is replaced with one volume of appropriate buffer. After incubation the amount of complement present in each solution (control and test) is determined by titration. The control sample should contain no less than 85 CH_{50} units. The value of complement present in the test sample (CH_{50}/ml) is subtracted from the control value, and no more than 20 CH_{50} units may be inactivated. In order to ensure a control value above 85 CH_{50}, the initial complement titration is performed on complement that has been heated. Therefore more than 100 CH_{50} units are actually present in all of the test and control samples. The release limit for this test is considered to be less than 20 CH_{50} units complement per ml of product.

Reactions

A reaction possibly mediated by spontaneous activation of the complement system was deemed to have occurred if any one or more of the following symptoms were present either during or up to four hours postinfusion: warmth, flushing, headache, fever, chills, anxiety, malaise, faintness, nausea, vomiting, muscle pains, abdominal cramps, chest pain, chest tightness, dyspnea, wheezing, tachycardia, or rash.

RESULTS

Reactions—Group 1

During the period under study, there were a total of ten possible complement-mediated adverse reactions (Table I).

Table I. Spontaneously reported possible complement-mediated adverse reactions in group 1, March 1986 through December 1987.

Lot Number	AC Activity (CH_{50}/ml) Method 1	Nature of Symptoms Reported
40B06	13.1	Chest tightness, dizziness, difficulty in breathing, lethargy
40B08	12.8	Headache after infusion
40P03	10.6	Vomiting, shortness of breath, abdominal pain after infusion
40P09	13.5	Generalized urticaria
40R02	14.2	Fever, increased pulse, chills, headache, general aches
40R03A	13.5	Flushing and fever after infusion
40S09 40S10	8.8 8.5	Dyspnea, hypotension, back pain, chills after infusion (patient received two lots)
Not known	Not available	Headache, fever, malaise ten hours after infusion
Not known	Not available	Wheezing, chest tightness, dyspnea during rapid infusion
Not known	Not available	Wheezing, chest tightness, dyspnea during rapid infusion

AC = anticomplementary.

No lot had more than one such reaction; however, lot numbers for three reactions were not recorded and therefore no data are available. The reactions did not seem to occur with greater frequency with lots having higher AC activity.

During the one-year, nine-month study period, over 200 lots of IGIV, pH 4.25 were released. The distribution of the AC activity in these lots is shown in the figure. AC values ranged from 5.6 to 17.2 units CH_{50}/ml. Most of the lots (80%) fell within the range 8.0 to 14.0 units CH_{50}/ml.

Reactions—Group 2

There were a total of 15 reactions, four of which occurred during infusion (Table II). Although one of the four reactions was considered severe, it did not necessitate discontinuing the infusion. Seven of the reactions, including the one classified as severe, were reported in association with the lot with low AC activity; all three reactions considered to be definitely related to the study drug infusion also occurred with this lot. There were five reactions with the lot with mid-range AC activity, and three reactions with the lot with high AC activity.

Comparison of Two Assays of Anticomplementary Activity

Ten lots of IGIV, pH 4.25 released for general usage were selected at random and their AC levels were compared using the two assays. The results are shown in Table III. It may be seen that according to

Percent of Total Lots

Figure. A
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Table II. I
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Lot Number
PR 3025
PR 3032
PR 3055

AC = anti
* Only t
† Reacti

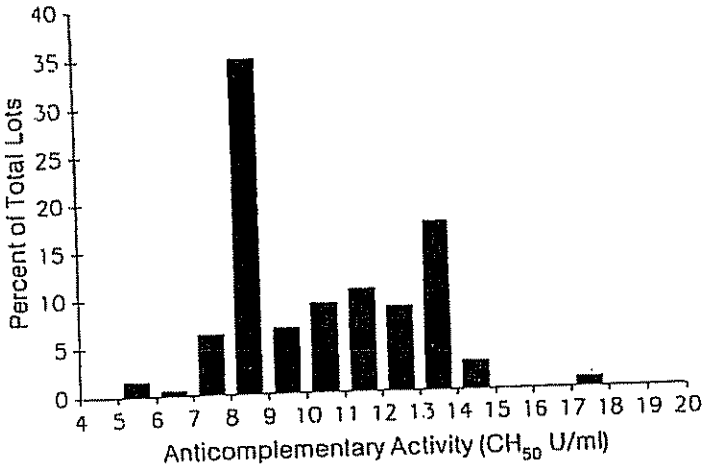

Figure. Anticomplementary activity of lots of IGIV, pH 4.25 between March 1986 and December 1987 (Method 1).

Table II. Possible complement-mediated reactions in group 2 during the surveillance study.

Lot Number	AC Activity (CH_{50}/ml) Method 1	Nature of Symptoms Reported
PR 3025	20.8	Rash after infusion Fever, lethargy after infusion Headache after infusion
PR 3032	15.6	Headache, seeing spots during infusion Abdominal cramps after infusion Headache after infusion Headache, abdominal cramps after infusion Headache after infusion
PR 3055	11.9	Chest tightness during infusion* Chest tightness, dyspnea during infusion*† Headache during infusion* Emesis after infusion Cough after infusion Emesis after infusion Emesis after infusion

AC = anticomplementary.
* Only three of the reported reactions were considered definitely drug-related.
† Reaction classified as severe.

Table III. Comparison of anticomplementary (AC) activity in ten randomly selected lots of IGIV, pH 4.25 by two AC assay methods.*

Lot Number	AC Activity (CH_{50}/ml)	
	Method 1	Method 2
40B04	11.9	25.3
40B09	12.8	23.4
40B10A	11.9	24.7
40B11	13.5	28.6
40C04	17.2	28.5
40C05	14.3	25.5
40C07	10.4	21.6
40C08C	7.7	23.3
40C13A	8.6	26.0
40D02	8.2	25.1

* With Method 1, an acceptable AC level is considered to be below 25 units CH_{50}/ml, while with Method 2 it is considered to be below 20 units CH_{50}/ml. Pearson product-moment correlation = 0.56 ($P = 0.09$).

Method 1, all lots were considered to have acceptable AC activity, while with Method 2, all lots fell outside the limit for AC activity. None of these lots was associated with any spontaneously reported reactions even though one lot had the highest AC activity of all the lots released during the period under review.

The Pearson product-moment correlation between the results obtained with the two assays was found to be 0.56, which is not statistically significant ($P = 0.09$).

DISCUSSION

Spontaneously reported reactions are undoubtedly an underestimation, while the reverse is probably true for the definitive study in which every possible symptom ever associated with a complement-mediated reaction was taken to indicate such a reaction. Many of the reactions may have been totally unrelated to the complement system, as is suggested by the absence of correlation between AC activity and the incidence of the reactions. In the definitive study, only one reaction was considered severe. There were no life-threatening reactions in either group of patients. There did not appear to be any correlation between the number and the severity of possible complement-mediated reactions with the level of AC activity in the lot used; in the definitive study there was almost a reverse correlation.

The data suggest therefore that the AC activity release limit and the assay used to measure the activity are reasonable and would not be associated with complement-mediated–type adverse reactions. The study showed that lots released in accordance with the specification limits defined by this assay did not show any significant incidence of complement-mediated adverse reactions.

The data also highlight the importance

of using an AC assay that is specifically designed and validated for the sample (eg, intravenous immunoglobulin) that is to be assayed. The Cutter Biological assay of AC activity (Method 1) used to release lots of IGIV was designed for IGIV, pH 4.25. When the assay of AC activity designed for a pepsin-treated intravenous immunoglobulin (Method 2) was used, all IGIV, pH 4.25 lots tested in this manner had AC activities that were well above the release cutoff limit. Yet none of these lots failing the AC limit in accordance with this assay were associated with any adverse reactions. Finally, there was no correlation between the assay of AC activity by Method 1 and by Method 2 when both were used to test AC levels in IGIV, pH 4.25.

CONCLUSION

This study clearly demonstrates that the AC activity levels in IGIV, pH 4.25 are acceptable as defined by an assay validated to measure the levels in this preparation. This is also reflected in the safety of the preparation by the virtual absence of possible complement-mediated–type adverse reactions. The assay validated for a preparation finished by treatment with pepsin was unsuitable.

ACKNOWLEDGMENTS

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Serum Fe Ingestion

Earl B. Daw

Department of Medical Bran

ABSTRACT

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EXHIBIT 26

(4)

PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

Patent MSB-7232

BACKGROUND OF THE INVENTION

Field This invention generally deals with an intravenously injectable immunoglobulin product, and more specifically deals with an intravenously injectable immune serum globulin (IGIV) which has been subjected to a virus inactivation step and which has a low level of anticomplement activity.

Background Early pharmaceutical preparations of immune serum globulins could not be administered intravenously due to an unacceptably high incidence of adverse reactions. These adverse reactions were associated with a decrease in serum complement levels, apparently caused by complement binding to the administered gamma globulin. (1) The ability of gamma globulin to bind complement, or its anticomplement activity (ACA), is greatly increased as a result of denaturation brought about during the fractionation procedure. Several approaches have been taken to address the problem of rendering ISG safe for intravenous administration. (See (2) and references therein). Tenold reported a method of preparing an immune serum globulin (ISG) with low ACA which could be administered by intravenous injection. (2, incorporated herein by reference). The Tenold '608 process requires formulating the ISG at low ionic strength (preferably less than about 0.001) and at low pH (3.5-5.0).

Other methods of preparing intravenously injectable immune serum globulin (IGIV) have been reported, including stabilizing with carbohydrates such as maltose (3). A process including incubation of ISG at pH 4.0 at 37° C (4) results in a product with low ACA which may be administered by intravenous injection; however, upon storage the product regains its high ACA. IGIV has also been prepared by covalent modification of the ISG, for example by proteolysis (5) or by reduction of disulfide linkages followed by reaction with a blocking agent (1,6).

Antibody preparations, since they are isolated blood products, have an inherent hazard of transmitting virally-mediated diseases. Inactivation of viruses is an important step in producing safe and effective blood products. U.S. Patent 4,540,573 to Neurath et al., which is incorporated herein by reference, describes a viral inactivation process using a trialkyl phosphate and detergent process (hereinafter, the solvent/detergent process, or SD process). (7) That solvent/detergent method has gained acceptance as being efficacious in the inactivation of lipid-enveloped viruses with limited adverse effects on biological activity or blood product profile. (8, 15; See also 12 for a discussion of various viral inactivation processes).

Current antibody preparations on the market generally have been regarded as safe with respect to viral contamination. (9) This is thought to be due to features of the fractionation processes used to isolate these blood products. However, it would be desirable to further ensure the safety of the antibody preparations by including a distinct viral inactivation step in the production process. Successful reduction of viral activity in an IGIV solution was reported using several different methods of viral inactivation for a variety of viruses. (16, 17) A process for preparation of immunoglobulins substantially free of retrovirus has been reported involving incubation of ISG under controlled conditions of time, temperature, and pH. The process entails isolating ISG via a cold ethanol plasma fractionation process and then storage of the ISG at one of two storage conditions: (a) at pH≤ 4.25 at a temperature of 27° C for at least three days, or (b) at pH ≤ 6.8 at a temperature of 45° C for at least six hours. (10).

We have found that using the SD process to treat ISG preparations, especially those subsequently formulated according to the Tenold '608 patent, results in a product with an acceptable viral inactivation but with unacceptably high levels of ACA. Elevated ACA levels were always detected at the sterile bulk stage (i.e., after compounding as 5% or 10% IGIV and filtration with 0.2 μm sterile filters) of all tri-n-butyl phosphate (TNBP)/detergent treated

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IGIV preparations regardless of process scale. Preparations of ISG with high ACA levels are not suitable for intravenous injection and instead must be administered via other routes, e.g. intramuscular (IM) injection. However, IGIV preparations are more desirable since they are immediately available in the bloodstream and are not subject to loss associated with IM injection. It is thus desirable to have an IGIV product which is both low in ACA and has been subjected to a viral inactivation step.

SUMMARY OF THE INVENTION

The invention is a method for producing an intravenously injectable immune serum globulin (IGIV) preparation with low anticomplement activity which has been chemically treated to render it substantially free of lipid-enveloped viruses. The method comprises a solvent/detergent viral inactivation step followed by an incubation step. We have discovered that the incubation step is necessary to achieve an acceptable level of ACA low enough to allow the ISG to be administered by intravenous injection. The incubation step should be conducted under controlled time, pH, temperature, and ionic strength. Preferably, the pH should be maintained between about 3.5 and about 5.0, the temperature should be within a range of about 2 to about 50° C, and the ionic strength should be less than about 0.001. In a preferred embodiment the ACA of the ISG preparation decreases gradually over a period of at least about ten days when the ISG is maintained at a pH of about 4.25 at low ionic strength (less than about 0.001) and the viral inactivation step (in a model system) results in a substantial reduction (i.e. at least 4 logs) in the titer of lipid enveloped viruses.

BRIEF DESCRIPTION OF THE FIGURE

The Figure shows a comparison of the typical average observed ACA levels of 5% IGIV solutions treated according to the SD process and with or without the follow-up incubation of the present invention.

SPECIFIC EMBODIMENTS

Materials and Methods

The starting material for the process of this invention is unmodified human immune serum globulin. In the specification and claims the term "immune serum globulin" is used to define the substance also referred to in the literature variously as gamma globulin, IgG and immunoglobulin G. It consists predominantly and preferably of at least about 85 percent of the 7S species of gamma globulin, which has a molecular weight of about 160,000. Any remainder is preferably 9S species, with a molecular weight of about 300,000. Both standard immune and hyperimmune serum globulins, e.g., tetanus, rabies and hepatitis immune serum globulins, can be employed, the solvent/detergent treated product being immune and hyperimmune ISG, respectively. Thus, a suitable starting material for the process of this invention is Cohn's Fraction II or Fraction III filtrate. (See Refs. 13, 14.)

Fraction II, by ultracentrifugation studies, is predominantly (about 85 percent) the 7S (sedimentation constant of 7) species of gamma globulin with an average molecular weight of 160,000. The remaining protein is essentially 9S material with a M.W. of about 300,000. Wet Fraction II paste (approximately 30 percent solids) is commonly lyophilized to obtain dry ISG powder which is then dissolved and prepared for intramuscular injection as a 16.5 percent sterile solution. Either the wet Fraction II paste or the dry ISG powder is a suitable starting material for the process of this invention.

Gamma globulin obtained by any process which has essentially the same composition of protein components as found in the Cohn Fraction II or Fraction III filtrate can be used as starting material in the present process. Both standard immune serum globulin and hyperimmune serum globulin can be employed as starting materials. As is well known, the

latter is produced from plasma or serum obtained from selected donors who have much higher titers for a specific antibody than is normally found in the average population. These donors have either been recently immunized with a particular vaccine or else they have recently recovered from an infection or disease. These high titer sera or plasmas are pooled and subjected to the usual Cohn fractionation procedures up to the point of isolating Fraction II.

Furthermore, because the amount of antibody required to achieve a desired immunological response is substantially less when administered intravenously, it will be apparent the intravenous dose will be substantially less than the intramuscular dose which will produce the same serum antibody titer. Thus, the dose of intramuscular ISG and hyperimmune serum globulin must be higher than that required to achieve the same serum antibody titer when globulin of the same antibody activity is administered intravenously.

The starting wet paste or lyophilized power is dissolved in a volume of water or other physiologically-acceptable carrier to provide a protein solution of a concentration of about 0.5-20% preferably about 5 to 10 percent. If Fraction III filtrate is employed, the aqueous solution must be concentrated by conventional techniques to the desired protein concentration. Any protein concentration may be used in this method; however, the above range is preferred from a practical standpoint.

After the protein has been dissolved or concentrated, the solution is adjusted to a pH of about 3.5 to 5.0 preferably about 3.8 to 4.2, by addition of a physiologically-acceptable acid such as hydrochloric acid. In general, the pH is adjusted to a point whereat the monomeric material in the protein solution is maintained at a maximum. However, the pH must not be so low as to result in gelation. The temperature should not be harmful to the ISG material. Good results are obtained within the temperature range of about 0 - 20° C. It is not necessary to hold the so-adjusted material for any period of time prior to the next step; however, the material may be held, if desired, without detrimental effects.

The protein solution at the appropriate pH (preferably 3.8 - 4.2) may be diafiltered with at least 4 volume exchanges of water to reduce the alcohol concentration from approximately 17% (Filtrate III) to about 2% alcohol. The efficacy of solvent/detergent as a viral inactivation method is much better at or above ambient temperatures; however, high concentrations of alcohol at these temperatures will denature the IgG molecules. Thus, this inactivation must be performed in low alcohol concentration.

Next, the protein concentration of the so-treated material is adjusted to the level desired for incubation with TNBP/detergent, generally less than 10% protein for maximum viral inactivation. This adjustment is accomplished by conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Prior to addition of TNBP/detergent, the pH may be adjusted within a wide range, depending on the detergent to be used. With Tween 80, the pH may be as low as 3.5, where the IgG starts becoming unstable. With cholate, the pH is adjusted to within the range of 5.0 - 6.4, preferably about 5.6, prior to addition of TNBP/detergent. Satisfactory cholate solubility during incubation was achieved by adjusting the immunoglobulin solutions to a pH of 5.5 or higher prior to addition of TNBP and sodium cholate. Adjusting the IgG solution to pH values lower than 5 5 is not suitable because the solubility of sodium cholate is highly dependent on pH (cholic acid pK = 6.4), with poor solubility at pH 5.5 or lower. Furthermore, maximum viral inactivation during incubation with TNBP/cholate was observed at pH values less than 6.0 in experiments which employed model viruses spiked into IgG solutions. The inactivation of HIV-1 and BVDV (bovine viral diarrhea virus, which is employed as a model for hepatitis C) was accelerated at pH 5.8, with inactivation to the detection limit occurring in 1-2 hours, whereas inactivation to the detection limit required a minimum of 6 hours when pH 7 conditions were used.

Next, the TNBP/detergent is added to the protein solution (preferably less than 8% [w/w], pH 5.8) mixed thoroughly, and then incubated above ambient temperatures, for example 30° C, with continuous agitation or mixing. Target TNBP/cholate levels for optimal viral inactivation during the incubation step should be > 3 mg/mL TNBP and > 2 mg/mL cholate as defined by Edwards et al. (8) Moreover, for effective viral inactivation, it is important that the solution is essentially free of particulates in order to facilitate thorough mixing of solvent/detergent and IgG solution. After incubation with TNBP/cholate under these conditions, greater than 5.2 $\log_{10}$ reduction of HIV-1 and greater than 4.0 $\log_{10}$ reduction of BVDV were detected.

After completing the incubation which provides the viral inactivation, the solvent and detergent molecules must be removed in order to achieve a final product with low levels of residual TNBP and cholate which would be suitable for intravenous administration. Generally, procedures to remove detergent are also effective in removing TNBP, and vice versa. Very low levels of TNBP and cholate in the final container can be achieved by a combination of filtration, diafiltration and hydrophobic chromatography. After completing the incubation, the majority of cholate (and TNBP) can be removed from the protein solution by filtration, providing the solution had been previously adjusted to a lower pH value such as 4.0, because sodium cholate is sparingly soluble in aqueous solutions at such pH values. Moreover, all processing steps which follow the solvent/detergent incubation are performed at lower pH values (i.e., 4.0) because IgG molecules are more stable at pH values between 3.5 - 5.0, in low ionic strength solutions. (2) Thus, after incubation with TNBP/cholate, the protein solution is adjusted to approximately pH 4.0 and incubated at 0 - 8° C in order to promote cholate precipitation. Next, filtration is employed to remove the precipitated cholate from the IgG solution.

The so-treated solution is diafiltered with at least four volume exchanges of water to reduce the ionic strength and to remove additional TNBP and cholate. After or during the above treatment, the pH is measured and maintained within the range of about 3.5 - 5.0. The protein concentration of the so-treated material is adjusted to 10 - 30%, usually 13% (w/v) by employing conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Again the pH of the preparation is maintained within the range of about 3.5 - 5.0, preferably about 3.8 - 4.2.

In the present invention, hydrophobic chromatography is employed to remove the TNBP and cholate not eliminated by the filtration and diafiltration steps, and thus provide a final product with low levels of residual TNBP and cholate which is suitable for intravenous administration. Hydrophobic chromatography is a method for TNBP removal from protein solutions that has fewer drawbacks and limitations than other available methods such as oil extraction, ion exchange or affinity chromatography. In part, this is because the protein of interest (IgG) remains in solution throughout the TNBP removal process. Polystyrene-based resins (typically PLRP-S from Polymer Laboratories, Amherst, MA) were used to remove the solvent/detergent from solution, as we have found the polystyrene-based resins to be superior to other resins, such as silica-based C-18 resins.

Next, the ISG preparation is adjusted to 5% or 10% protein, and treated to render it tonic, i.e., to render it compatible with physiological conditions, or render it physiologically acceptable upon injection. In a preferred embodiment, the tonicity is adjusted to about 230 to about 490 mosmol/kg solvent. More preferably, the tonicity range is from about 250 to about 350 mosmol/kg solvent, and most preferably the tonicity range is from about 260 to about 325 mosmol/kg solvent. The 5% formulation (5% IGIV) is made tonic by the addition of 10% maltose. The 10% formulation contains 0.2 M glycine in order to achieve an isotonic preparation without large quantities of sugar. The product with either formulation

(Gamimune®N 5% or Gamimune®N 10%) experiences shifts in molecular distribution (antibody aggregation) when the ionic strength of the low pH solution is increased. Therefore, sodium chloride, which is often used to achieve tonicity, should not be used.

The so-treated solution is incubated at pH 4.25 under low ionic strength conditions (NLT 21 days at 20 - 27° C preferred) in order to provide a lowering of ACA levels. The ionic strength is determined according to Perrin (18), and in a preferred embodiment the ionic strength should be less than about 0.001. Elevated ACA levels were always detected at this stage of all TNBP/cholate treated IGIV preparations (regardless of process scale); however, ACA levels are gradually lowered by incubation at pH 4.25 under low ionic strength conditions (Tables 3, 5 - 7). While there is no strict rule for determining when the ACA level is low enough to be an acceptable level suitable for intravenous administration, IGIV preparations should have ACA levels as low as possible.

The Figure depicts the typical average reduction of ACA observed in 5% IGIV solutions following SD treatment. For a 5% ISG formulation the acceptable level suitable for intravenous administration preferably would be less than about 45 CH_{50} units/mL, and more preferably less than about 30 CH_{50} units/mL. For a 10% ISG formulation, the acceptable level suitable for intravenous administration preferably would be less than about 60 CH_{50} units/mL, and more preferably less than about 45 CH_{50} units/mL. As used herein, one unit of ACA activity (one CH_{50} unit) is defined as the amount of protein capable of activating 50% of the complement in an optimally titered complement and red blood cell/hemolysin system. The assay measures the amount of complement that is bound by the mixture of standardized amounts of complement and protein. See refs. 19-20 for a discussion of the assay. Briefly, red blood cells that have been sensitized by preincubation with red blood cell antibodies are added to the complement/protein mixture. In the presence of free complement (not already bound by the protein) these sensitized cells will lyse, releasing hemoglobin which can be quantitated as a measure of the degree of lysis. In parallel, sensitized red blood cells are also added to a buffer control-complement mixture, whose degree of lysis is defined as 100%. The

difference between the actual amount of complement needed to give 100% lysis and the amount of complement remaining unbound in the presence of protein equals the amount of complement actually bound by the protein, or anticomplement activity.

Results

Anticomplement activity of ISG resulting from viral inactivation process

To establish the effect of the SD viral inactivation process on solutions containing ISG which are formulated according to the Tenold '608 patent, the experiments depicted in Table I were performed. The starting material (SM) was Cohn process filtrate III which had been ultrafiltered to about 5% protein and then diafiltered with four volumes of water.

In the control experiment, incubation (-)/SD (-), the SM was not subjected to any incubation or solvent/detergent treatment. In the incubation (+)/SD (-) experiment, the pH of the SM was adjusted to 7.0, the solution was incubated at 30° C for ten hours, and then the pH was reduced to 4.0. In the incubation (+)/ SD, TNBP & Tween 80 (+) experiment, the pH of the SM was adjusted to 7.0, 3 mg/mL TNBP and 2 mg/mL Tween 80 were added to the solution, the solution was incubated at 30° C for ten hours, and then the pH was reduced to 4.0. In the incubation (+)/SD, TNBP & cholate (+) experiment, the pH of the SM was adjusted to 7.0, 3 mg/mL TNBP and 2 mg/mL cholate were added to the solution, the solution was incubated at 30° C for ten hours, and then the pH was reduced to 4.0. The solutions in each experiment were then diafiltered with four volumes CWFI (cold water for injection) and concentrated by ultrafiltration. After addition of dry maltose to 10% w/v, the 5% IGIV solution (pH 4.25) was filtered through a 0.2 μm filter.

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Table 1
Anticomplement activity in 5% IGIV produced by variations of the Solvent/Detergent IGIV Process

	ACA (CH_{50}/mL)
Control (no solvent/detergent treatment, no 30° C incubation)	25
Incubate at 30° C for 10 hr (no solvent/detergent)	22
Incubate at 30° C for 10 hr NLT 3 mg/mL TNBP NLT 2 mg/mL Tween 80	68
Incubate at 30° C for 10 hr NLT 3 mg/mL TNBP NLT 2 mg/mL cholate	> 100

* These samples were assayed for ACA after final compounding according to the Tenold '608 patent, but they were not incubated at pH 4.25 and 22° C prior to analysis.

The results listed in Table 1 show that levels of ACA increased in IgG samples after incubation with TNBP/cholate or TNBP/Tween 80. ACA levels were not elevated in IgG samples that were incubated for 10 hr at 30° C in the absence of solvent/detergent. These results suggest that ACA levels of IGIV samples were not elevated by either processing manipulations or incubation for 10 hr at 30° C in the absence of solvent/detergent.

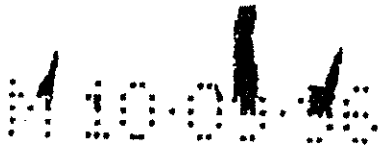
Table 2
Anticomplement activity in 5% IGIV spiked with TNBP/Na cholate

	ACA (CH_{50}/mL)
5% IGIV, no TNBP/cholate	12
5% IGIV with 100 μg/mL TNBP, 100 μg/mL Na cholate	13

Furthermore, spiking experiments (with TNBP and Na cholate, Table 2) have demonstrated that the elevated anticomplement activity levels were not artifacts caused by carrying out the anticomplement assay in the presence of trace levels of TNBP/Na cholate. Thus, using the prior art SD process for viral inactivation of a solution containing ISG, subsequently formulated according to the Tenold '608 patent, yields a product which has high ACA and is unsuitable for intravenous administration. In a similar experiment, SD treated samples which were not incubated (Table 3, Initial Testing) had ACA levels greater than 100 units.

Table 3
Reduction in Anticomplement activity of samples previously treated with TNBP/cholate

Sample	ACA (CH_{50}/mL)	
	Initial Testing (no incubation)	After incubation 6 wk. @ 5°C 3 wk. @ 22°C
RB21872-16	> 100	33
RB21872-17	> 100	34
RB21872-18	> 100	36
RB21872-20	> 100	27



However, when duplicate SD treated samples were incubated for extended periods of time (6 weeks at 5° C and 3 weeks at 22° C), the level of ACA was markedly reduced (Table 3, after incubation). This led to further investigation of this surprising observation.

Aggregate content of ISG exposed to TNBP/cholate

The samples of the previous experiment (Table 3, Initial Testing) were analyzed by size exclusion (gel permeation) HPLC immediately after compounding to determine the extent of aggregation of the IGIV at the initial time point. HPLC analysis shows nearly complete monomer content in the samples. (Table 4).

Table 4
HPLC analysis of non-incubated 5% IGIV samples (Table 3 Initial)

Sample	Aggregate (%)	Dimer (%)	Monomer (%)	Fragment (%)
RB21872-16, initial	0.140	0.00	99.86	0.00
RB21872-17, initial	0.146	0.00	99.85	0.00
RB21872-18, initial	0.124	0.00	99.88	0.00
RB21872-20, initial	0.172	0.00	99.83	0.00

Previously, high IgG aggregate levels were shown to correlate with high anticomplement activity. However, results from analysis of the samples show the level of ACA in the samples to be greater than 100 units. (Table 3, 'Initial Testing') The HPLC analysis shows that the high ACA following the TNBP/cholate treatment was not due to the presence of aggregated IgG molecules.

Varied conditions of time and temperature

The SM was the same as in the previous experiment, and experimental conditions were similar with the following changes. The solutions were treated with TNBP/cholate at pH 7.0 and then were compounded to 5% IGIV, 10% maltose, pH 4.25, as above. The ACA was assayed immediately after final compounding, after a first incubation for nine days at 5° C, and after a second incubation for 21 days at either 22° C or 5° C. The results are presented in Table 5.

Table 5
ACA of TNBP/cholate treated IGIV samples

Sample Point	ACA (CH_{50}/mL)
Intermediate Samples	
Initial sterile bulk	> 100
Incubated 9 d. @ 5°C	> 100
Final Incubation	
21 d. @ 22°C	49
21 d. @ 5°C	71

In the initial sterile bulk sample, which was treated with TNBP/cholate at pH 7.0, the level of ACA was again greater than 100 units for the initial time point, confirming the observations noted in Table 3. Upon incubation at 5° C for nine days, the ACA remained greater than 100 units. The final incubation step at either 5° C or 22° C shows that the reduction in ACA is dependent on temperature, with faster reduction in ACA observed at higher temperatures.

Effect of pH during solvent/detergent treatment on ACA

ACA levels were evaluated after incubation with TNBP/cholate at pH 5.8 because better viricidal activity was observed at pH values less than 6.0. Generally, the non-incubated

sterile bulk samples of material incubated at pH 5.8 had lower ACA levels than the pH 7.0 samples, but the trend of lowering ACA upon incubation was repeated in the pH 5.8 samples. In fact, the ACA levels continue to decrease beyond the 21 day incubation in samples that initially had elevated ACA levels after incubation with TNBP/cholate at pH 5.8 (Table 6). As was previously noted for the samples incubated at pH 7.0, the lowering of ACA was not due to decreasing levels of aggregated IgG molecules because the material treated at pH 5.8 was essentially monomeric IgG prior to 22° C incubation (HPLC analysis, sample A4, Table 8).

Table 6
Sample A4 - ACA upon extended incubation

Incubation at 22°C (days)	CH_{50}/mL
0	122
10	73
19	55
25	56
28	45
30	40
34	39
41	33
48	30
55	29

Similar results were achieved with samples formulated to 10% IGIV, 0.2 M glycine in the sterile bulk stage. Upon incubation at low ionic strength at pH 4.25 for 10 and 21 days, the levels of ACA were seen to decline in both 5% IGIV samples and 10% IGIV samples. (Table 7) The decrease in ACA can thus be observed over a range of ISG concentrations and over a range of pH values for the solvent/detergent treatment. (Tables 3, 5, 7) HPLC analysis (Table 8) of the sterile bulk samples presented in Table 7 confirmed that the elevated ACA levels were not due to aggregation of ISG molecules.

Table 7
ACA of samples treated with TNBP/cholate at pH 5.8

Sample	Sterile bulk (day zero) (CH_{50}/mL)	10 days incubation at 20 - 27°C (CH_{50}/mL)	21 days incubation at 20 - 27°C (CH_{50}/mL)
A1 (5% IGIV)	43	ND	10
A2 (5% IGIV)	31	14	15
A3 (5% IGIV)	44	15	12
A4 (5% IGIV)	122	73	55
B1 (10% IGIV)	>100	48	46
B2 (10% IGIV)	49	36	30
B3 (10% IGIV)	53	ND	37

Taken together, the above results suggest that ISG products which have been subjected to a solvent/detergent viral inactivation process resulting in an undesirable ACA increase can be made suitable for IV administration by incorporating an additional incubation step under the conditions described here to reduce the ACA to an acceptable level.

Table 8
HPLC Analysis of sterile bulk samples treated with TNBP/cholate at pH 5.8

Sample	Aggregate (%)	Dimer (%)	Monomer (%)	Fragment (%)
A2	0.140	0.00	99.86	0.00
A3	0.146	0.00	99.85	0.00
A4	0.124	0.00	99.88	0.00

CONCLUSION

The ACA increase resulting from the solvent/detergent treatment of the IGIV (antibody) solution appears to be an unavoidable secondary effect of TNBP/detergent treatment to inactivate viruses in the solution. I have discovered that by incubating the solution of IGIV at low pH (4.25) and low ionic strength (0.001) for a relatively long period of time (at least about 10 days), the ACA gradually decreases over the period of incubation.

The prior art discloses a method of producing IGIV (the Tenold '608 patent) using low pH and low ionic strength. The Tenold '608 method omits the viral inactivation step, and thus avoids the problem of increased ACA, but the possibility of viral activity remains. Unlike Tenold, incubation is an essential aspect of the present invention for reducing the ACA.

The Neurath et al. '573 patent teaches the solvent/detergent viral inactivation step. However, Neurath '573 does not mention controlling the pH and also does not mention any consequences of the process relating to ACA. Elevated ACA levels were detected at the sterile bulk stage of TNBP/cholate treated IGIV preparations. However, ACA levels decreased upon

M 10-09-96

incubation for at least about 10 days at pH 4.25, low ionic strength, and not less than about 20° C. (See Tables 5-7) The prior art describes several approaches to lowering ACA levels of purified IgG preparations, including removal of IgG aggregates. (11) IgG aggregates have been shown to activate the complement system in vivo. (1) In the present invention, however, lowering of IgG ACA was not due to decreasing levels of IgG aggregates because these TNBP/cholate treated IGIV preparations contained low levels of aggregated IgG (as measured by HPLC, Tables 4, 8) prior to incubation under such conditions.

It would be desirable to produce substantially virus-free IGIV, but following the prior art results in a product with an unacceptable level of ACA. Note that Tenold '608 states that the product is substantially free of ACA, but use of the SD process in conjunction with Tenold '608 does result in high levels of ACA: experimental results reported here show that treating ISG solutions with the SD process and then formulation according to the Tenold '608 patent leads to a product with high ACA. (See Tables 1, 3, 5-7) The surprising finding reported here is that a follow-up (terminal) incubation step lowers the ACA of the solvent/detergent treated solution. The typical average observed ACA levels of 5% IGIV solutions treated according to the SD process and with or without the follow-up incubation are compared in the Figure. The present invention thus includes a previously unobserved method of reducing the ACA by incubating under controlled conditions of pH, temperature, and ionic strength for a period of time, thus allowing the product to be administered by intravenous injection.

Mitra '714 does not suggest the use of a S/D process but, instead, reports that a relatively brief incubation of an ISG product under similar conditions results in a substantially virus free preparation. (10) However, employing incubation under such conditions to provide a lowering of anticomplement activity is a novel application of these incubation conditions which were previously employed in the IGIV process for inactivation of enveloped viruses.

The newly developed IGIV process reported here, which includes an additional internationally accepted viral inactivation procedure (treatment with TNBP/cholate), generates IgG preparations which have low ACA levels and are suitable for IV administration. The major advantage is that an IGIV product with improved safety can be obtained by a two-step process that includes a TNBP/cholate treatment for viral inactivation and incubation under conditions that afford low ACA levels that are suitable for IV administration.

The above disclosure is intended to illustrate the invention, and it is thought variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention should be limited only by the claims below.

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M 10-09-96

(5)

CLAIMS

What is claimed is:

1. A method of treating a solution of antibodies which may have virus activity, the method comprising

   a) contacting the solution with a trialkylphosphate and a detergent under conditions sufficient to substantially reduce any virus activity and resulting in a given level of anticomplement activity; and

   b) then incubating the solution of step a) under conditions of controlled time, pH, temperature, and ionic strength, such that the anticomplement activity of the solution is reduced to an acceptable level suitable for intravenous administration.

2. The method of claim 1, wherein the anticomplement activity is reduced to less than about 60 CH_{50} units/mL.

3. The method of claim 1, wherein the solution comprises about 5% wt./wt. antibody and the anticomplement activity is less than about 45 CH_{50} units/mL.

4. The method of claim 3, wherein the solution comprises about 5% wt./wt. antibody and the anticomplement activity is less than about 30 CH_{50} units/mL.

5. The method of claim 1, wherein the solution comprises about 10% wt./wt. antibody and the anticomplement activity is less than about 60 CH_{50} units/mL.

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6. The method of claim 5, wherein the solution comprises about 10% wt./wt. antibody and the anticomplement activity is less than about 45 CH_{50} units/mL.

7. The method of claim 1, wherein the incubation is for at least about ten days.

8. The method of claim 1, wherein the pH is maintained within a range of about 3.5 to about 5.0.

9. The method of claim 1, wherein the temperature is maintained within a range of 2° C to 50° C.

10. The method of claim 1, wherein the ionic strength is less than about 0.001.

11. The method of claim 1, wherein at least about 99% of the antibodies are monomeric.

12. The method of claim 1, comprising the further step of adjusting the tonicity of the solution to a physiologic value under such conditions that the ionic strength is not appreciably altered.

13. The method of claim 12, wherein the tonicity of the solution is adjusted by adding a carbohydrate to the solution.

14. The method of claim 13, wherein the carbohydrate used is maltose.

15. The method of claim 12, wherein the tonicity of the solution is adjusted to a range of about 230 to about 490 mosmol/kg solvent.

M 10-09-95

16. The method of claim 15, wherein the tonicity of the solution is adjusted to a range of about 274 to about 309 mosmol/kg solvent.

17. The method of claim 12, wherein the tonicity of the solution is adjusted by adding an amino acid to the solution.

18. The method of claim 17, wherein the amino acid used is glycine.

19. The method of claim 1, wherein the trialkylphosphate is tri-n-butyl phosphate and the detergent is selected from polysorbate 80 and sodium cholate.

20. The method of claim 1, wherein the solution has a pH between about 3.5 and about 6.0 during step a).

21. An intravenously injectable immune serum globulin preparation produced by the method of claim 1 and substantially free of lipid enveloped viruses, wherein the preparation has an ionic strength less than about 0.001, a pH between about 3.5 and about 5.0, an antibody concentration of about 5% wt./wt., and a maltose concentration of about 10% wt./wt.

22. The preparation of claim 21, wherein the pH is about 4.25.

23. An intravenously injectable immune serum globulin preparation produced by the method of claim 1 and substantially free of lipid enveloped viruses, wherein the preparation has an ionic strength less than about 0.001, a pH between about 3.5 and about 5.0, an antibody concentration of about 10% wt./wt., and a glycine concentration of about 0.2 M.

24. The preparation of claim 23, wherein the pH is about 4.25.

EXHIBIT 27

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European Patent Application No. 96 114 439.1-2113

With reference to the phone conversation I herewith file a new set of claims we agreed upon.

This set of claims may form the basis for an allowance.

There is deemed to be no need for an Oral Proceedings anymore.

(Dr. Frank Burkert)
General Authorization No. 18552)
Enclosure

Enclosure: EP App. No 96114439.1-2113
MSB 7232EP/2002-02-21

CLAIMS

1. A method for the preparation of an antibody solution having low viral activity and low anticomplement activity, the method comprising:

   a) contacting a first antibody solution with a trialkylphosphate and a detergent until the titer of the lipid enveloped viruses present in the first antibody solution is reduced by at least $4\log_{10}$ to produce a second antibody solution;

   b) removing trialkylphosphate and detergent from the second antibody solution to produce a third antibody solution, and

   c) incubating the third antibody solution for a period of at least ten days at a pH maintained between 3.5 and 5.0, a temperature within a range of 2°C to 50°C, and at an ionic strength of less than 0.001 to produce the antibody solution having low viral activity and low anticomplement activity.

2. The method of claim 1, wherein the anticomplement activity of the antibody solution is less than 60 CH_{50} units/ml.

3. The method of claim 1, wherein the antibody solution comprises 5% wt./wt. antibody and has an anticomplement activity of less than 45 CH_{50} units/ml.

4 The method of claim 3, wherein the antibody solution comprises 5%wt./wt. antibody and the anticomplement activity is less than 30 CH_{50} units/ml

5. The method of claim 1, wherein the antibody solution comprises 10% wt./wt. antibody and has an anticomplement activity of less than 60 CH_{50} units/ml

6. The method of claim 5, wherein the antibody solution comprises 10% wt./wt. antibody and has an anticomplement activity of less than 45 CH_{50} units/ml.

7. The method of any of claims 1 to 6, wherein at least 99% of the antibodies in the antibody solution are monomeric.

8. The method of claim 1, wherein between steps b) and c), tonicity of the third antibody solution is adjusted to a physiologic value under such conditions that the ionic strength of the third antibody solution is not appreciably altered.

9. The method of claim 8, wherein the tonicity of the solution is adjusted by adding a carbohydrate to the third antibody solution.

10. The method of claim 9, wherein the carbohydrate is maltose.

11. The method of claim 8, wherein the tonicity of the third antibody solution is adjusted to a range of 230 to 490 mosmol/kg solvent.

12 The method of claim 11, wherein the tonicity of the third antibody solution is adjusted to a range of 274 to 309 mosmol/kg solvent.

13 The method of claim 8, wherein the tonicity of the third antibody solution is adjusted by adding an amino acid to the third antibody solution.

14. The method of claim 13, wherein the amino acid is glycine.

15. The method of any of claims 1 to 14, wherein the trialkylphosphate is tri-n-butylphosphate and the detergent is selected from polysorbate 80 and sodium cholate.

16. The method of any of claims 1 to 15, wherein the first antibody solution is contacted with trialkylphosphate and detergent at a pH between 3 5 and 6.0.

EXHIBIT 28

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EXHIBIT 29

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EXHIBIT 30

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EXHIBIT 31

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